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Hardeep Singh

University of Connecticut - Storrs, hsingh2014@gmail.com

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Oxygen Supplementing, Biocompatible Outer Membranes for Enhanced Performance of Implantable Glucose Sensors

University Scholar Thesis

Submitted By

Hardeep Singh

Physiology & Neurobiology

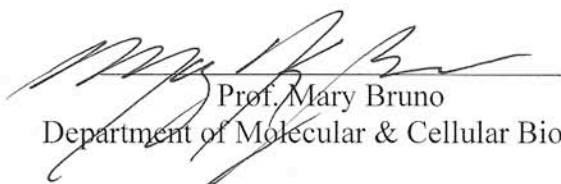
Class of 2010



Prof. Fotios Papadimitrakopoulos
Department of Chemistry



Prof. Joseph Crivello
Department of Physiology & Neurobiology



Prof. Mary Bruno
Department of Molecular & Cellular Biology

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Chapter 1: Introduction

Diabetes Mellitus is a metabolic disorder that affects the pancreas thereby causing an imbalance in blood glucose⁴. There are two types of diabetics, type I diabetics have a problem with producing sufficient amounts of insulin and type II diabetics have an insensitivity to insulin. For optimal diabetes care, it is very important to monitor blood glucose. The conventional method of monitoring blood glucose involves pricking the finger and drawing blood onto a test strip; this is a very inconvenient way of keeping track of blood glucose. By contrast, a glucose sensor that is implanted in the body (and capable of communicating with a personal electronic accessory) could alleviate a diabetic from the painful process of periodic finger pricking².

Glucose sensors based on various detection techniques (like electrochemical⁵, optical¹⁹, etc.) have been developed. However, the specificity and selectivity of electrochemical-based glucose detection rendered it a popular choice for implantable glucose sensors⁵. While the research has been progressing rapidly, a state of the art prototype is not available to date. This has been attributed to various issues including low sensitivity, narrow dynamic range, enzyme degradation, biofouling, etc¹⁷.

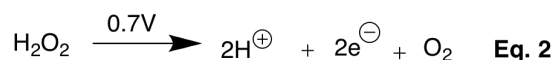
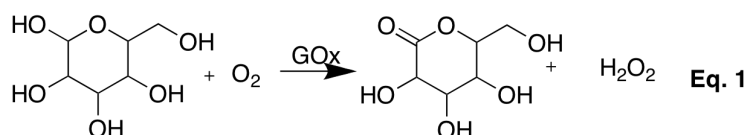
To overcome these complications researchers have employed various outer membranes such as Humic acids/ferric cations (HAs/Fe³⁺)⁵, HAs/poly(diallyldimethylammonium chloride) (PDDA)⁵, poly(styrene sulfonate) (PSS/PDDA)⁵, poly(vinyl alcohol) (PVA) hydrogels¹⁷, hydrophilic polyurethane (HPU) membrane mixed with polyvinyl alcohol/vinyl butyral copolymer (PVAB)¹⁹, pullulan acetate (PA)/polyethylene glycol (PEG)/heparin²⁰. The problem with using outer membranes is that they overcome the complication of biocompatibility at the expense of increasing the response time of sensors. A different outer membrane for improving response time and a different membrane for improving biocompatibility are required. This

increases the number of outer membranes required and increases the response time of the sensors. Can we make a single membrane that simultaneously improves linearity and biocompatibility but at the same time reduce response time?

To address this question, we fabricated glucose biosensors that utilize poly-vinyl alcohol (PVA) hydrogels to simultaneously improve linearity and biocompatibility. The choice of PVA hydrogels is based on prior publications from our lab, where PVA has been used as a matrix for drug delivering moieties^{5,23}. The project focused on the following specific aims: 1) Optimization of enzyme immobilization technique and 2) Optimization of PVA membranes.

1.1. Background & Significance

The concept of using an enzyme based glucose sensors was first introduced by Clark and Lyons in 1962². They used glucose oxidase (GOx) as the glucose sensing enzyme and based their measurements according to equation 1 as shown below:



Further advances in glucose sensing were made by the Yellow Spring Instrument Company in 1975 when they launched a glucose analyzer which could measure the amount of glucose present in a very small approximately 25µl sample of blood². Updike and Hicks employed two oxygen electrodes to monitor glucose levels⁶. They immobilized GOx on one of the electrodes to measure the concentration of glucose in biological solutions⁶. In 1973, Guilbault and Lubrano introduced amperometric monitoring of glucose in which they immobilized GOx with cellophane and monitored the production of hydrogen peroxide (H₂O₂) in

the above reaction and calibrated it with the amount of glucose present⁷. This calibration was based on the Eq. 2 (mentioned above).

Despite these efforts, the problem of oxygen acting as the limiting reactant exists. Since oxygen is involved in the reaction (Eq. 2), it can lead to saturation of the sensors because the physiological concentration of oxygen in blood is very low compared to glucose. Saturation occurs when the sensor is incapable of sensing further glucose. This has been overcome by using mass-transport limiting films or designing sensors which only allow the influx of glucose in one direction and the influx of oxygen from both directions. Alternatively oxygen rich carbon pastes can also be used¹²⁻¹³. The advantage of these mass-transport films is that they regulate inwards glucose diffusion to prevent sensor saturation¹².

A second problem with measuring the current produced by the oxidation of H_2O_2 is that the measurement is also affected by the oxidation of electroactive interferent species such as ascorbic acid, uric acid, and acetaminophen. To exclude these currents the sensors are coated with permselective membranes, which exclude interferent species based on size, charge, or polarity⁸⁻¹¹. Poly(*o*-phenylenediamine) (PPD) and polypyrroles permselective coatings have shown the best ability to eliminate interferences while still allowing the enzyme layer to work⁸⁻⁹.

1.2. Objectives

Our specific aims focused on the fabrication of glucose biosensors, which overcome the issues of sensitivity, response time, and saturation while at the same time tackling the issue of biocompatibility. These sensors were designed to effectively counteract the oxidation of interferent species. *O*-phenylenediamine (OPD) was electropolymerized onto the sensors to form PPD (figure 3.1) and used as the interferent-blocking membrane because it has been shown to

have the best ability at counteracting interferent species without affecting the sensing-enzyme GOx²².

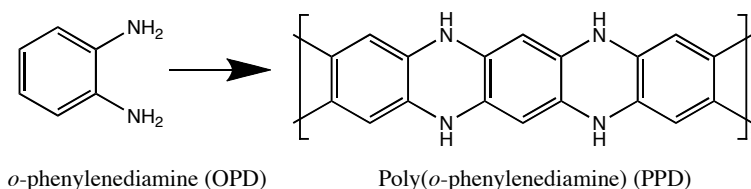
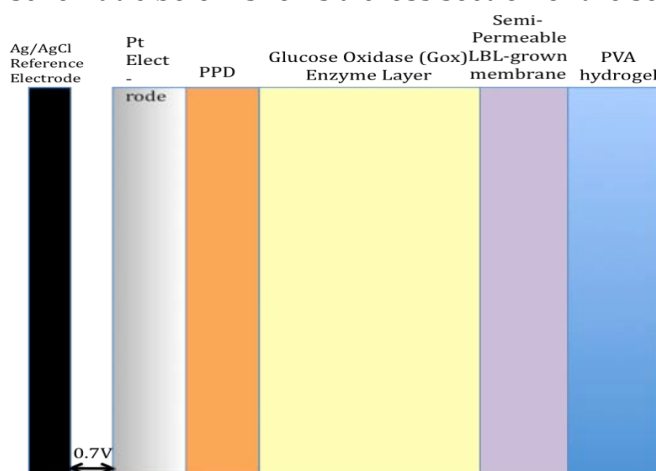


Figure 3.1: Conversion of OPD to PPD

GOx was used as the glucose-sensing enzyme, which carried out the oxidation of glucose to produce glucorolactone and H_2O_2 . The H_2O_2 was further oxidized at the platinum electrode and the current generated was measured using a reference electrode. This current was be calibrated with the appropriate concentration of glucose present. The reaction occurring at the GOx layer is represented by equations 1 and 2 (shown above).

One of the main parts of this project entailed coating the sensors with (PVA) hydrogels to overcome the issue of biocompatibility. PVA hydrogels have been used in drug or protein delivery systems, and contact lenses due to their soft, permeable, and hydrophilic interfaces with the body¹⁶. The close resemblance of the hydrogels to human tissue makes them an ideal candidate for use in glucose sensors, allowing for both optimum efficiency and biocompatibility²³. The PVA hydrogels were cross-linked onto the sensors. Cross-linking of PVA hydrogels onto the sensors involves repetitive freezing and thawing cycles¹⁶. Following fabrication of the sensor, its performance needs to be evaluated to meet standard criteria. The schematic below shows a cross section of the sensor:



Chapter 2: Sensor Performance Criteria

Various factors need to be considered in order to develop optimal implantable glucose biosensors. The sensor needs to be biocompatible so that the body does not elicit an immune response against the sensor and cause inflammation and rejection of the sensor. The enzyme component of the sensor needs to have long-term stability of the enzyme so that it does not degrade over time and stop functioning. The sensor also needs to be designed so that it shows great sensitivity and linearity in measuring glucose. Linearity is the ability of the sensor to sense increasing concentrations of glucose; the current obtained from the sensor over time should show a linear relationship, to indicate a large dynamic range.

After fabrication the sensors are tested using the CHI1000A Series Multi-Potentiostat in a phosphate-buffered saline (PBS) solution (pH \approx 7.4) at 37°C under an applied constant potential of 0.7V vs. an Ag/AgCl reference electrode. Glucose selectivity tests are performed by administering increasing concentrations of glucose every hundred seconds, following a 500 second stabilization period, throughout a 2300 second time period. The glucose concentrations range from 2mM-130mM, going well beyond the physiological range of glucose. The amperometric responses of the sensors were fitted to a Michaelis-Menten equation to obtain the

respective $K_{m,Glu}^{app}$ and K_{m,O_2}^{app} using the following equation:
$$J_{glu} = \frac{J_{max}}{\frac{K_{m,O_2}^{app}}{[O_2]} + \frac{K_{m,glu}^{app}}{[Glu]}}.$$

The linearity of the sensors were determined by plotting sensor response current vs. the glucose concentration and determining the $K_{m,Glu}^{app}$. The linear range sensitivity (LRS) of the sensor was determined as the slope of the amperometric current density (J_{Glu}) vs. glucose

concentration: $LRS = \frac{J_{max}}{K_{m,Glu}^{app}}$. The oxygen dependence of these sensors was performed by sealing

the chamber containing the three electrodes (working electrode, Ag/AgCl reference electrode, and Pt counter electrode) along with a commercial oxygen sensor (Mettler Toledo, Model 4100e). Glucose was administered and the chamber was purged with nitrogen to remove the desired amount of oxygen and response of the sensor as a function of oxygen concentration was obtained for various glucose concentrations.

Interferent tests are performed by administering increasing concentrations of the respective interferent species: ascorbic acid, uric acid, and acetaminophen, throughout a 1700 second time period. The slope of the amperometric current density for each of the interferent species vs. its concentration is calculated, allowing for the determination of the selectivity of the sensor for glucose. The selectivity of each individual sensor is calculated as follows:

$$\%S_{AA} = \frac{J_{AA}}{J_{Hydrogen\ Peroxide}} \times 100$$

$$\%S_{UA} = \frac{J_{UA}}{J_{Hydrogen\ Peroxide}} \times 100$$

$$\%S_{AP} = \frac{J_{AP}}{J_{Hydrogen\ Peroxide}} \times 100$$

$$\text{Total selectivity: } 100\% - (\%S_{AA} + \%S_{UA} + \%S_{AP})$$

Chapter 3: Materials

This chapter lists all of the materials used in this study:

1. Glucose oxidase enzyme (E.C. 1.1.3.4, 157,500 units/g, *Aspergillus niger*)
2. Glutaraldehyde [25(w/v)% solution in water]
3. *Ortho*-phenylenediamine (PPD)
4. Bovine serum albumin
5. Phosphate-buffered saline (PBS)
6. Sodium salt of HA (molecular mass 169 kDa)
7. Ferric chloride hexahydrate (reagent grade)
8. D-glucose (reagent grade)
9. Poly(sodium 4-styrenesulfonate)(PSS)(Mw 70,000)
10. Poly(diallyldimethylammonium chloride)(PDDA) [20(wt)% in water; Mw 200,000–350,000]
11. PVA (99% hydrolyzed, molecular mass 133 kDa)
12. Platinum (Pt) wires
13. Silver wires
14. Deionized water was produced by a Millipore (Milli-Q) system with resistivity >18 M
15. Electrochemical analyzer (Model CHI1000A Series Multi-Potentiostat)

Chapter 4: Optimization of Enzyme Immobilization Technique

4.1. Background & Significance:

The coating of PPD films onto the Pt electrodes is carried out via the electropolymerization of OPD in an acetate buffer, as described earlier. PPD has been widely used in the field of biosensors for its ability to entrap enzyme membranes and selectively allow the permeation of various analytes, as well as block the permeation of others of interest, especially the interferent species²⁴. PPD has been shown to block oxidation of interferent species such as ascorbic acid, uric acid, and acetaminophen, which are likely to oxidize at the operating potential of the sensor. The electropolymerization of OPD to PPD may be carried out by varying pH which alters its structure and changes its oxidation potential²⁴. By increasing the pH of the acetate buffer, it has been shown that the oxidation potential is shifted cathodically²¹. Varying the pH of the acetate buffer used to electropolymerize OPD also contributes to changes in the functional groups which further contribute to the degree of conjugation in the resultant PPD structure²⁴. This degree of conjugation is important because it dictates how easy it will be to excite an electron into the lowest unoccupied molecular orbital's (LUMO), dictating the conductivity of the membrane²⁴.

In our studies, PPD was specifically used due to its various properties and its ability to provide the lowest sensitivity to interferents ($S_{\text{Interferents}}$) while providing a high sensitivity to H_2O_2 . ($S_{\text{H}_2\text{O}_2}$). This provides us with a membrane which will be optimal for use in glucose biosensors because it will avoid the oxidation of unwanted interferent species and allow the oxidation of glucose and H_2O_2 .

4.2. Objectives:

This objective for this part of the project was to determine the optimal environment for the electropolymerization of OPD. Previous studies have shown the optimal concentration of OPD used during electropolymerization to be 5mM⁵. In order to determine the optimal environment, the concentration of OPD was held constant at 5mM and the pH of the acetate buffer was varied.

Working electrodes (sensors) were fabricated by winding a 50 μ m platinum (Pt) wire on a 0.3mm diameter monofilament nylon line, yielding a total surface area of 3mm² for the working electrode. The working electrodes were then electrochemically cleaned in a 0.5M sulfuric acid (H₂SO₄) solution via potential cycling between -0.21 and 1.25V. The pH solutions were made using acetate buffer and equilibrated to their respective pH using a pH meter. A 5mM OPD solution was purged with nitrogen prior to electropolymerization. To carry out the electropolymerization on the sensors, a 1mL solution of 5mM OPD was used.

The working electrodes were then electropolymerized to yield a PPD membrane. The electropolymerization was carried out in an aqueous acetate buffer by applying a constant potential of 0.7V vs. a saturated calomel reference (SCE) electrode for 900seconds. The electrodes were then subsequently washed with distilled water and dried. The electrodes were left alone for a number of hours before being tested against interferent species.

Three interferent species (ascorbic acid, uric acid, and acetaminophen) were used to carry out the test along with hydrogen peroxide. The working electrodes were tested using the CHI1000A Series Multi-Potentiostat in a phosphate-buffered saline (PBS) solution (pH \approx 7.4) at 37°C under an applied constant potential of 0.7V vs. an Ag/AgCl reference electrode. After a stabilization period of 500 seconds, increasing concentrations of the interferent species were

administered. Figure 4.1 depicts the interferent test and the electrode response to increasing concentrations of interferent species.

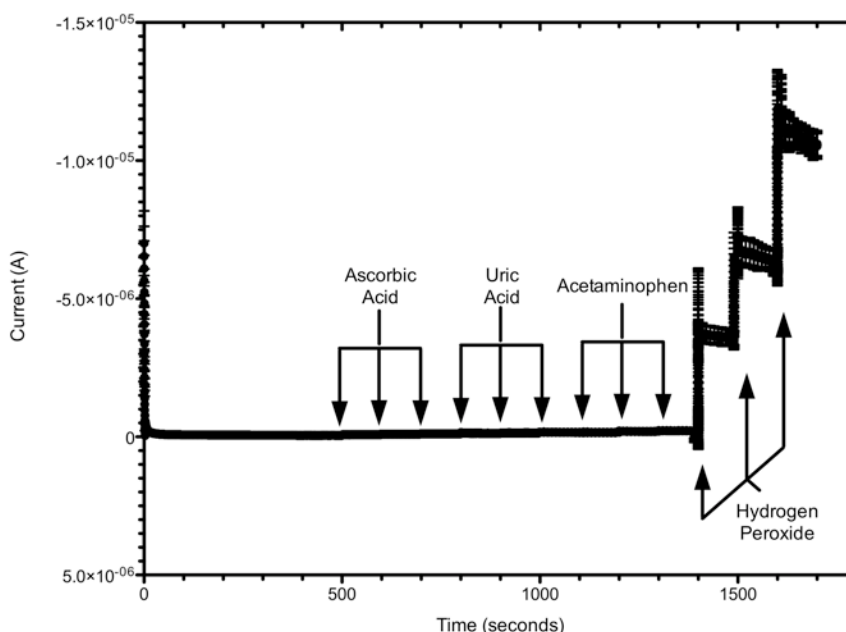


Figure 4.1: The figure above shows the CHI file obtained during the interferent test. The sensor was allowed a stabilization period of 500 seconds, after which increasing concentrations of three interferent species were added: ascorbic acid, uric acid, and acetaminophen. Afterwards, hydrogen peroxide was added and the sensitivity of each interferent species was calculated.

The data obtained from the tests was then analyzed using Microsoft Excel, allowing for the calculation of sensitivity for each interferent species as well as the total selectivity of the working electrode. In order to calculate the sensitivity for each interferent species, data points were obtained from the CHI files and plotted to obtain the slope (Current vs. Interferent concentration). This gave us the sensitivity of the electrode for the respective interferent species. The sensitivity for each species was calculated and subtracted from the sensitivity of the electrode for hydrogen peroxide. This allowed for the calculation of the total selectivity of the electrode.

4.3. Results and Discussion:

Data analysis allowed for the generation of Figure 4.2, which showed the percent selectivity for each pH used. It was observed that when the working electrode was fabricated

using a pH 5 buffer, it showed a 99% selectivity, up from 97% when fabricated using a pH 7 buffer. A general trend was observed, which showed pH 5 to have greatest selectivity, and as the pH increased or decreased the percent selectivity decreased. This was seen when the pH was decreased to 3, which showed a 90% selectivity, and also when it was increased to pH 9 (77%) and pH 11 (62%).

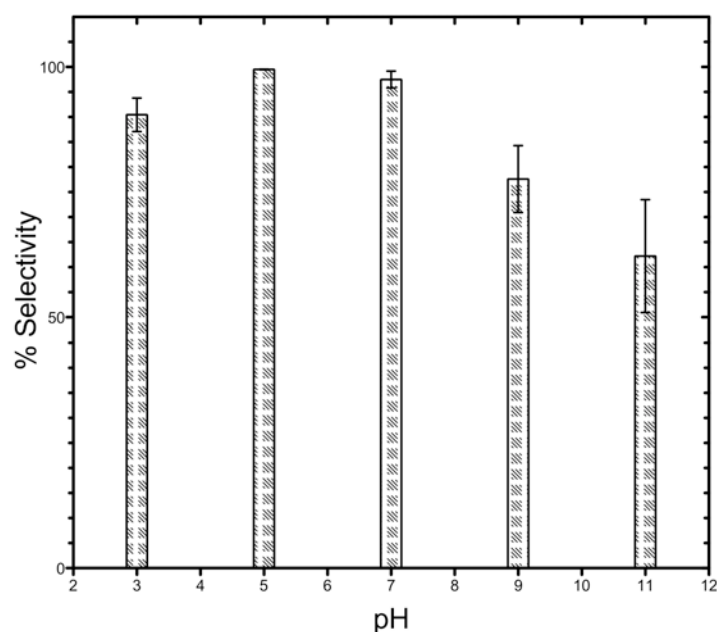


Figure 4.2: The figure shows the selectivity results from varying the pH of the buffer used to electropolymerize OPD onto the working electrodes. It can be seen that pH 5 shows the highest percent selectivity compared to other pH values. Standard deviation is shown using error bars and can be seen pH 5 has the lowest standard deviation.

The next step involved the incorporation of the glucose oxidase enzyme membrane in the sensor. Initial sensor fabrication with the PPD and GOx membranes yielded sensors of the Pt/PPD/GOx configuration. Testing and data analysis produced figure 4.3 which showed the response current vs. the glucose concentration. It is important to note that only the hatched area in Figure 4.3 is the physiological glucose range. As can be seen from figure 4.3, the response current of the sensor increases with increasing glucose concentration before saturating at higher glucose concentrations.

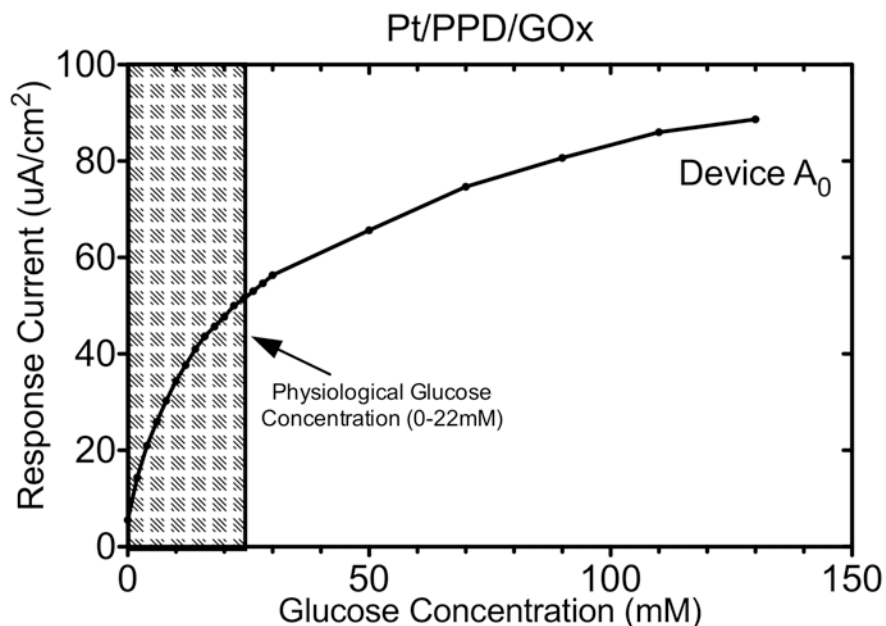


Figure 4.3. The figure shows the response current vs glucose concentration for sensors with no outer membranes. The hatched region illustrates the physiological range of glucose.

4.4. Conclusions:

To optimize the enzyme immobilization technique, the first step in the process was to optimize the environment in which the OPD is coated onto the sensor. Previous studies have shown that changing the pH of the OPD solution used during electropolymerization changes its structure and its degree of conjugation which affect the conductivity of the resultant PPD membrane²⁵. This is important for the proper functioning of the sensor due to the electron transfer that occurs between the enzyme and the PPD membrane.

Various working electrodes were synthesized at different pHs using a 5mM concentration of OPD, which was reported as optimal from previous studies^{5,23}. Interferent tests were performed using three interferent species and the results were analyzed to determine the percent selectivity of the working electrodes. It was determined that when the electropolymerization of

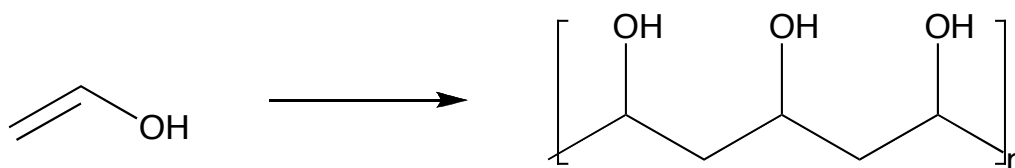
OPD is carried out in a pH 5 acetate buffer, a 99% selectivity is achieved. As the pH increases or decreases, the percent selectivity decreases with the lowest at pH 11.

This part of the project allowed us to determine the optimal environment for the electropolymerization of OPD onto the working electrodes. This provided us with a design which will allow us to fabricate sensors with very high sensitivity for hydrogen peroxide and a very low sensitivity for interferent species. This will prevent any unwanted oxidation of various species, which affect the sensor performance.

Chapter 5: Optimization of PVA membranes

5.1. Background & Significance:

Poly-vinyl alcohol (PVA) is a water-soluble polymer with varying properties ranging from film-forming, emulsifying, as well as adhesive properties²⁵. PVA membranes can be formed by electrospinning PVA due to its unique properties in forming fibers²⁵. PVA is a hydrophilic compound so it can be easily modified and incorporated with various proteins and substrates²⁵. Other ways to form PVA membranes are to dip coat the PVA onto a surface and subject it to a number of freeze-thaw cycles, which will cross-link the PVA forming hydrogels^{23,25}. PVA has the unique property of being very compact with a relatively low conductivity, making it ideal for its use in our sensors^{23,25}. The structure of PVA is shown below:



5.2. Objectives:

Previous studies using various semi-permeable membranes coated onto the sensor via a LBL-assembly process showed that 5 bilayers ($N=5$) of HAs/ Fe^{3+} were optimal for use in our sensors^{5,23}. This membrane provided the sensor with a high $K_{m,\text{Glu}}^{\text{app}}$, LRS, and a low $K_{m,\text{O}_2}^{\text{app}}$ ⁵. The high $K_{m,\text{Glu}}^{\text{app}}$ indicated its ability to detect increasing concentrations of glucose without saturation; the high LRS indicated its ability to work over a wide range of glucose concentrations and its versatility with increasing concentrations of glucose⁵. The low $K_{m,\text{O}_2}^{\text{app}}$ indicated its low dependency on oxygen, which is ideal for our sensor, because a low $K_{m,\text{O}_2}^{\text{app}}$ will allow the reactions shown in equation 1 and 2 to effectively proceed without any worry about limiting

reagents⁵. A low K_{m,O_2}^{app} does not limit the sensor in terms of when it will saturate due to low oxygen partial pressure⁵. Therefore, for all future experiments, unless mentioned otherwise, HAs/Fe³⁺ was used as the LBL-assembled membrane.

The next step was to incorporate PVA hydrogels in the glucose biosensors. Before coating our sensors directly with PVA, swelling experiments were carried out. The focus of this experiment was to determine the behavior of the PVA hydrogels in solution and their water uptake capacity. This is important because a number of properties of PVA depend on humidity and dictate the ability of PVA to uptake water^{23,25}.

To carry out the swelling experiments, a 10% (w/v) aqueous solution of PVA was prepared using 99% hydrolyzed PVA. The PVA was heated to 80°C to allow for proper dissolution and polymer assembly. The PVA was allowed to cool at room temperature before being used further. Small volumes of PVA were transferred to small vials, and the freeze-thaw cycles were carried out. A freeze-thaw cycle consists of freezing the sample for 3 hours in a freezer at -20°C, followed by a 1 hour thaw period at room temperature. Each vial was subjected to a different number of freeze-thaw cycles, ranging from 1 through 7.

To start the swelling experiments, the initial mass of the PVA (M_0) was obtained and the PVA was immersed in 20mL of PBS. The vials containing the PVA were immersed in a water bath held at a constant temperature of 37°C. The weight of the PVA was monitored every few hours, and was determined by removing the PVA from the vial and removing the excess PBS using a Kimwipe. This weight was recorded as M_t and the percent weight change of PVA was determined by the following equation: $(M_t - M_0)/M_0 * 100$. The weight of the PVA was monitored as a function of time over a 60 hour time period.

The next part of the experiment involved fabricating sensors with the configuration: Pt/PPD/GOx/LBL and coating them with PVA. Working electrodes (sensors) were fabricated by winding a 50 μ m platinum (Pt) wire on a 0.3mm diameter monofilament nylon line, yielding a total surface area of 3mm² for the working electrode. The working electrodes were then electrochemically cleaned in a 0.5M sulfuric acid (H₂SO₄) solution via potential cycling between -0.21 and 1.25V. The pH of the OPD solution used was 5 and the concentration of OPD was 5mM. The OPD solution was purged with nitrogen for 45 minutes prior to electropolymerization. The electropolymerization was carried out in aqueous acetate buffer by applying a constant potential of 0.7V vs. a saturated calomel reference (SCE) electrode for 900seconds. The electrodes were then subsequently washed with distilled water and dried.

The GOx enzyme membrane was consequently immobilized by dip coating the Pt/PPD electrode in a solution of 5mg/ml GOx followed by crosslinking with 50% (w/v) glutaraldehyde and 5mg/ml BSA. The electrodes were washed with distilled water and allowed to dry.

Five-bilayers of HAs/Fe³⁺ were coated onto for the LBL-assembled membrane, as described elsewhere⁵. This yielded devices A₅ (5 = number of bilayers), which were then coated with PVA, attaining device A₅P_N (N= number of freeze-thaw cycles). The sensors were tested for glucose, and the $K_{m,Glucose}^{app}$, LRS, and K_{m,O_2}^{app} ⁵ were determined. Data analysis allowed us to determine the optimal number of freeze-thaw cycles to use in our sensors.

Next, all of the three-semipermeable membranes were used to see how the addition of PVA affected sensor performance, yielding devices A₅, B₅, C₅. Oxygen studies followed to determine the oxygen storing capacity of the PVA hydrogels. As reported in previous studies, PVA hydrogels have been shown to form water-free hydrophobic domains that act as physical cross-links, storing oxygen²³. In order to confirm this, the oxygen content of the gels was

analyzed using a sealed chamber with a known amount of dried PVA in a glass tube under vacuum. The PVA was gelled through the freeze-thaw process described earlier²³. The PVA was then flame sealed in a glass tube and placed in an airtight chamber with 50mL of deionized water along with a commercial oxygen sensor²³. The chamber was purged with nitrogen to remove the oxygen in the chamber and the glass tube was crushed²³. The oxygen level of the gel was measured using the oxygen sensor. This was repeated as a function of freeze-thaw cycles²³.

5.3. Results & Discussion:

The swelling experiments were carried out over a time period of 60 hours. The main objective of this was to determine how much the PVA hydrogels would swell when incorporated into glucose biosensors. Figure 5.1(A) showed the percent weight change of PVA as a function of time, and Figure 5.1(B) showed the weight saturation as a function of freeze-thaw cycles.

It was observed that water uptake increased with immersion time up until 3 hours, after which the water uptake saturated for all of the freeze-thaw cycles. The water uptake by the gels was inversely proportional to the number of freeze-thaw cycles; therefore 7 freeze-thaw cycles showed the lowest water uptake while 1 freeze-thaw cycle showed the greatest water uptake. It was determined that water uptake was related to the extent to which the PVA crosslinked, thus dictating porosity and fluid uptake. The greater the number of freeze-thaw cycles, the more the PVA crosslinked and reduced the porosity, thus lower water uptake and lower weight change, and vice-versa. Figure 5.1(B) showed that as the number of freeze-thaw cycles increased, the saturation weight change decreased, which was related to the crosslinking of the PVA as well as the decreased porosity. Comparing figure 5.1(B) with the oxygen content of the PVA hydrogels, reported elsewhere, it can be observed that the saturation weight change is inversely related to the oxygen content of the PVA hydrogels²³. The optimization of the sensors requires a low K_{m,O_2}^{app}

and a high $K_{m,Glucose}^{app}$, can be achieved by optimizing the number of freeze-thaw cycles. It was determined from the swelling experiments that to allow for proper PVA swelling, it needs to be immersed in PBS for approximately 3 hours.

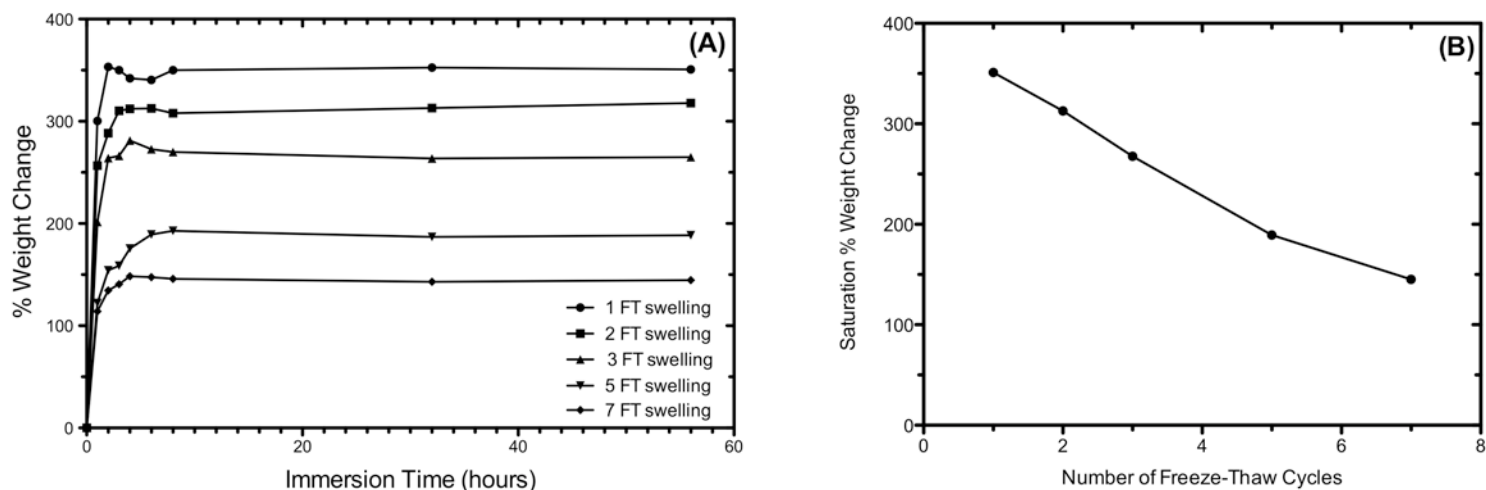


Figure 5.1. (A) Percent weight change of PVA hydrogels with various number of freeze-thaw (FT) cycles immersed in PBS buffer (pH 7.4) at room temperature as a function of the number of immersion time. (B) Saturation percent weight change of PVA hydrogel as a function of the number of freeze-thaw cycles.

Following the swelling experiments, devices A_5P_N were fabricated and tested, with N ranging from 1 to 7 freeze-thaw cycles. In order to coat the PVA on top of the sensors, the PVA was gelled through a freeze-thaw process, which causes partial dehydration and the formation of ordered hydrophobic structure via physical cross-links²³. As the number of freeze-thaw cycles increases so does the formation of the hydrophobic structures²³. Sensors were fabricated with the results obtained from earlier studies with the following configuration: Pt/PPD/GOX/LBL ($N=5$). These devices were then subjected to glucose testing to analyze the behavior of the sensors after PVA. Before testing was commenced, the sensors were immersed in PBS for three hours to allow for proper swelling of the PVA membrane, which allowed for optimal glucose flux. This was determined through PVA swelling experiments, as described above. This was important because immersion in PBS allowed the PVA to swell and open the pores in its

membrane to allow for adequate analyte flux. Data analysis was carried out in Microsoft Excel using the data obtained from the CH instrument, allowing for the determination of $K_{m,Glu}^{app}$, J_{Glu} , and LRS.

Figure 5.2 shows the variation in sensor linearity (A), sensor response current (J_{max}) (B), as well as the oxygen-slope of these devices (C) as a function of the number of freeze-thaw cycles. It can be clearly seen that the $K_{m,Glu}^{app}$ increases for the first two freeze-thaw cycles and then steadily decreases thereafter, with J_{max} increasing for the first three freeze-thaw cycles and dropping thereafter, figure 5.2A and 5.2B. Analyzing the data, it was determined that three freeze-thaw cycles showed the greatest J_{max} , indicating it has the greatest sensitivity to glucose. The sensors fabricated with three freeze-thaw cycles showed the second highest $K_{m,Glu}^{app}$, indicating their high linearity for sensing glucose. Through this data analysis, three freeze-thaw cycles were adopted as the optimum number of freeze-thaw cycles for our sensors. The drop in both the $K_{m,Glu}^{app}$ and J_{max} observed after three freeze-thaw cycles was attributed to the characteristic of the PVA gels. It was determined that as the number of freeze-thaw cycles increased the porosity of the gel was reduced, limiting the flux of glucose, which accounts for the lower $K_{m,Glu}^{app}$ and J_{max} ²³.

Figure 5.2C showed that as the number of freeze-thaw cycles increased, the oxygen slope decreased for the first three freeze-thaw cycles and then started to rise again. The oxygen slope data showed the dependency of the sensors on oxygen. The lower the oxygen slope the lower the dependency of the sensor on oxygen. In an ideal glucose sensor, the oxygen slope should be as low as possible to attain a high LRS. This would allow the sensor to work over a wide range of glucose concentrations without saturation. The data presented in Figures 5.2A, 5.2B, and 5.2C allowed us to conclude that three-freeze thaw cycles are optimal for use in our sensors.

In the next step, the oxygen content of the gels were analyzed and it was determined that the oxygen content increases as a function of freeze-thaw cycles (data not shown)²³. In fact, the oxygen content of 7 freeze-thaw cycles was three times greater than that of 1 freeze-thaw cycle²³.

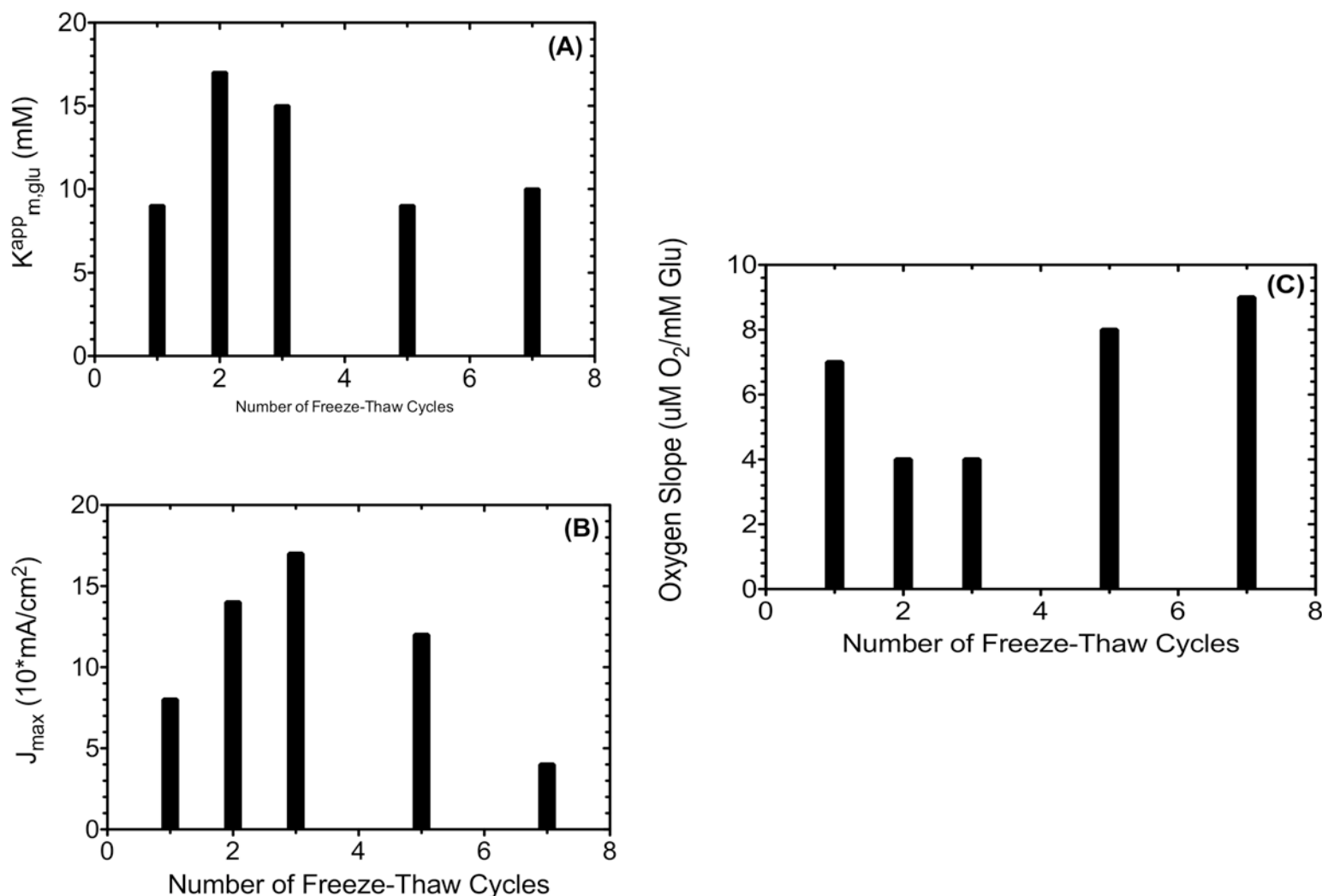
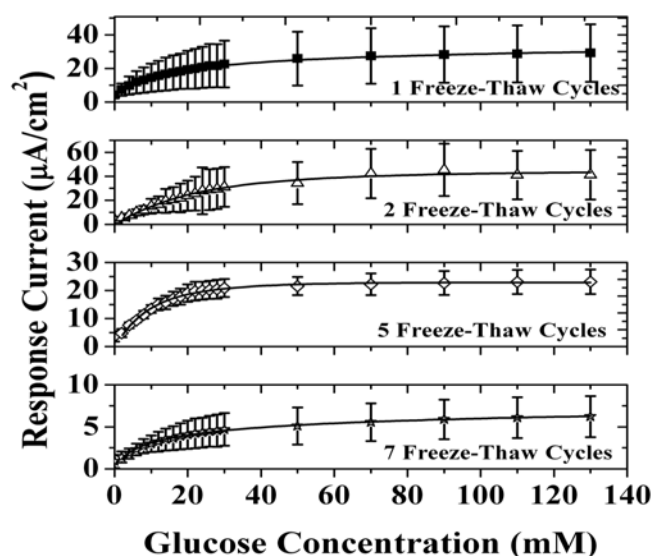


Figure 5.2. Variation of (A) sensor linearity ($K_{app_{m,glu}}$), (B) response current, (C) oxygen slope ($K_{app_{m,O_2}}$) as a function of the number of freeze-thaw cycles for sensors coated with (HA/Fe³⁺)/5/PVA hydrogel outer membranes.

Testing the sensors against increasing glucose concentrations allowed for the production of Figure 5.3 which depict the sensor response to increasing concentrations of glucose and figure 5.4, which shows the oxygen dependence as a function of glucose concentration. Figure 5.3 shows the amperometric currents of sensors fabricated with 5-bilayers of HAs/Fe³⁺ and various

freeze-thaw cycles of PVA as a function of glucose concentrations. The data for three freeze-thaw cycles is shown in Figure 5.6A. It can be clearly seen as before that the sensor response to increasing concentrations increased linearly before saturating at higher glucose concentrations.

Figure 5.4 shows the oxygen dependence of the five-bilayer sensors of HAs/Fe³⁺ and various freeze-thaw cycles of PVA as a function of glucose concentrations.



Figures adopted from prior publication “Enhanced Glucose Sensor Linearity Using Poly(Vinyl Alcohol) Hydrogels”

Figure 5.3: This figure shows the sensor response current vs glucose concentration as a function of time. The device used here had the configuration of Pt/PPD/GOx/LBL/PVA_N. The LBL membrane used here was 5 bilayers of HAs/Fe³⁺²³.

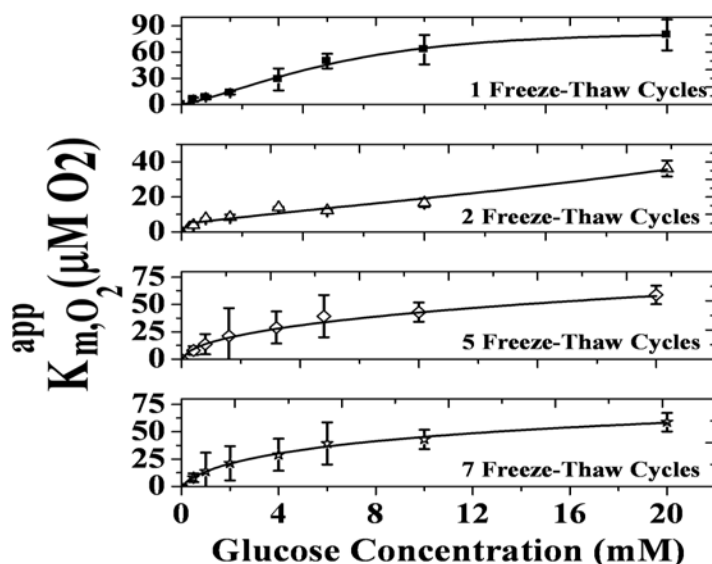


Figure 5.4: This figure showed the K_{m,O_2}^{app} as a function of glucose concentration. This allowed us to determine how dependent the sensor on oxygen²³.

These experiments and the data obtained allowed us to conclude that three freeze-thaw cycles were optimal for our sensors because they provided: (1) a high K_{m,O_2}^{app} , indicating their great linearity; (2) the greatest J_{max} , indicating their high responsiveness to increasing concentrations of glucose; and (3) a lower oxygen slope and sufficient oxygen storing capacity than any other number of freeze-thaw cycles. At the end of these experiments, the sensors had attained the configuration illustrated in figure 5.5.

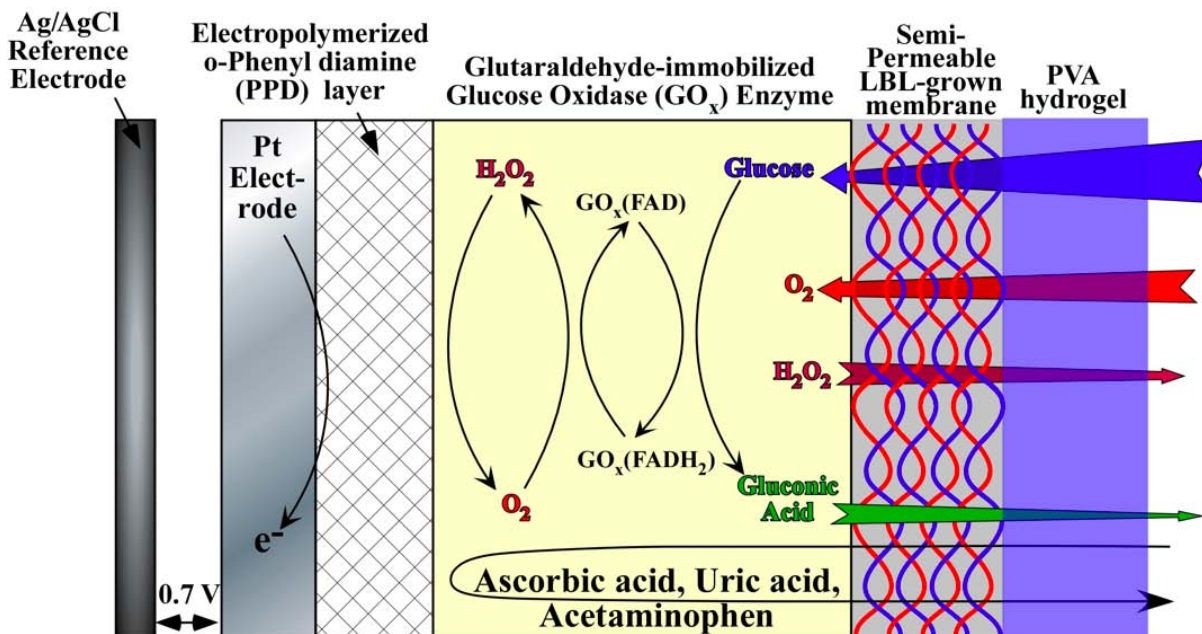


Figure 5.5. Schematic cross section of the glucose sensor in study²³.

In order to determine the effect of the PVA coating on the sensor's response, all three of the semi-permeable membranes were used to determine the most effective with the incorporation of PVA. The three semi-permeable membranes that were used were (A) HA/Fe³⁺, (B) HA/PDDA, and (C) PSS/PDDA. Three batches of sensors were fabricated with their respective outer semi-permeable LBL-grown membranes coated with PVA through three freeze-thaw cycles and tested. At this point the sensors had obtained the configuration depicted in Figure 5.5.

Data was obtained and the response current of the sensors vs. the glucose concentration was plotted as shown in Figure 5.6. Figure 5.6 allowed us to analyze all three semi-permeable membranes. Comparing Figure 5.6 with the results obtained in previous studies using LBL-assembled membranes without PVA, it was easily noted that with a layer of PVA on top of the sensors, the response current was reduced^{5, 23}. This was attributed to the reduced glucose flux towards the enzyme membrane due to the reduced porosity of the PVA membrane, which in limiting glucose flux, thereby reduced the response current²³.

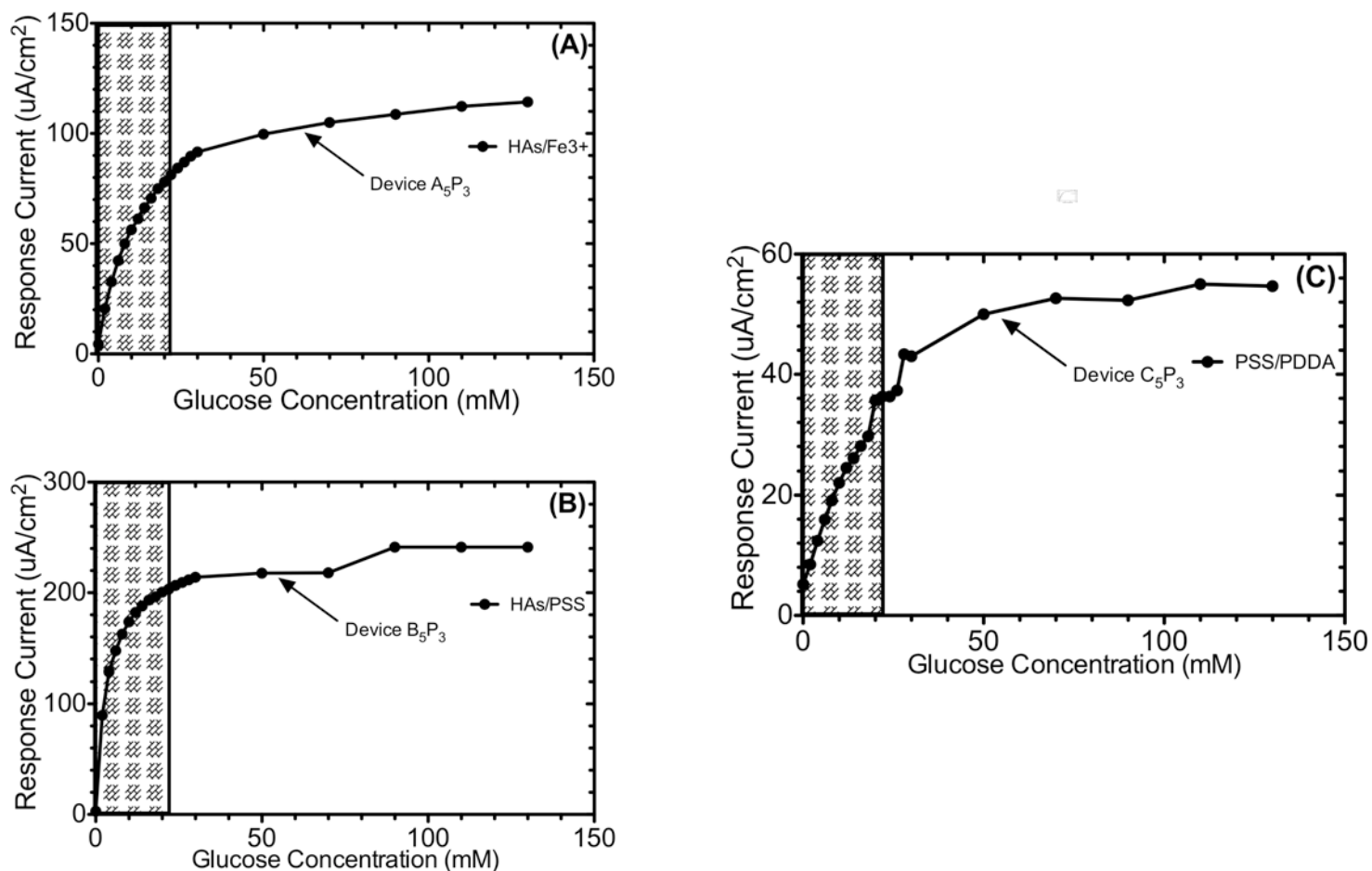


Figure 5.6. The figure shows the amperometric current vs glucose concentration for sensors containing five bilayers of (A) HA/Fe³⁺, (B) HA/PDDA, and (C) PSS/PDDA semipermeable membranes after deposition of PVA hydrogels, and subjected to three freeze–thaw cycles.

Figure 5.7 was also obtained which provides a closer view of the hatched portion of figure 5.6, showing the sensors response to physiological concentrations of glucose. Figure 5.7 shows that the overall response current to increasing concentrations of glucose decreases when the sensors are coated with PVA. It was easily discerned that sensor or device A₅P₃ showed the greatest linearity after PVA coating as compared to other devices. This indicated the strong sensitivity of device A₅P₃ towards glucose. As can be seen with all the sensors in figure 5.7, the response of the sensors increases with increasing concentrations of glucose and eventually saturating at their respective $K_{m, \text{Glu}}^{\text{app}}$.

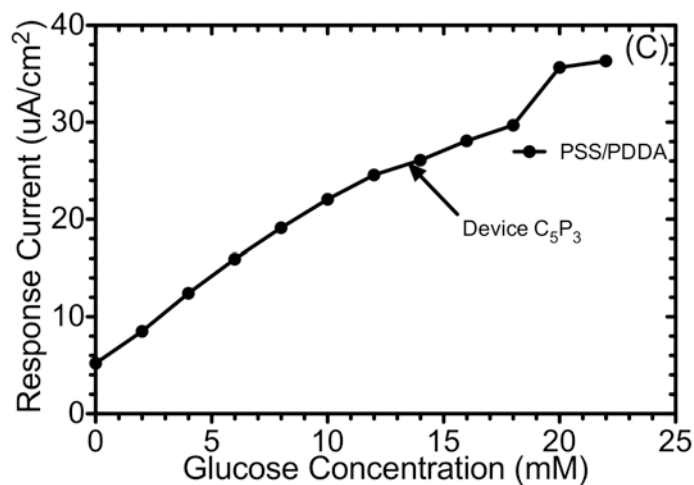
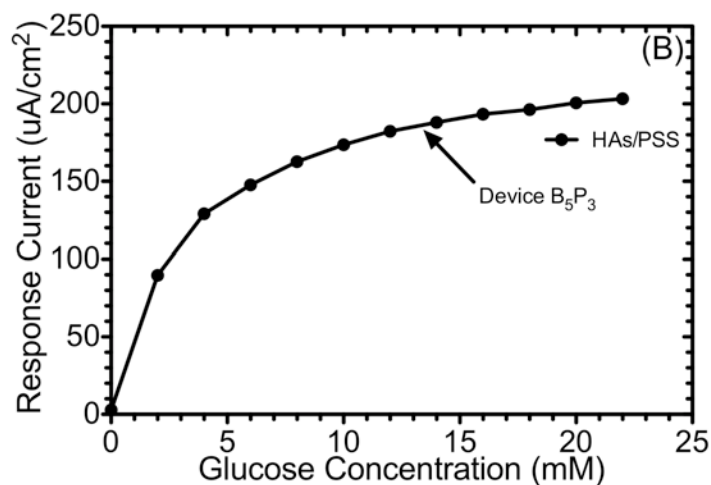
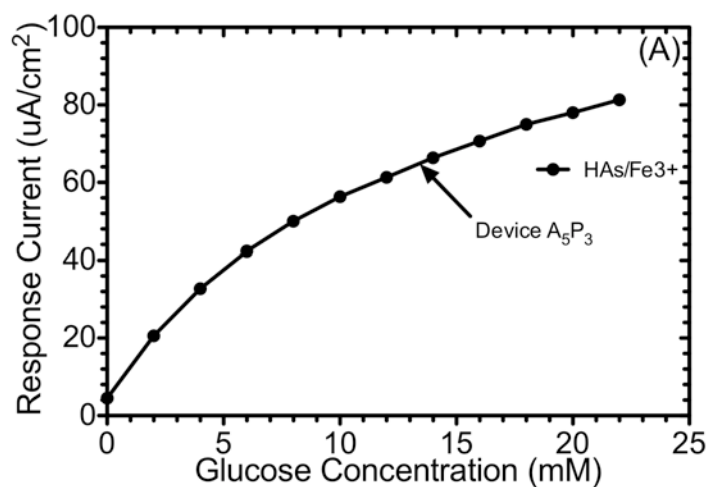


Figure 5.7. The figure shows the sensor response current vs. glucose concentration (physiological range) for sensors contained five layers of (A) HA/Fe³⁺, (B) HA/PDDA, and (C) PSS/PDDA semipermeable membranes, before and after deposition of PVA hydrogels, subjected to three FT cycles.

Glucose sensitivity is important but become useless if the sensor is highly dependent on oxygen, because as the oxygen concentration declines as a function of time, the sensor will cease to function effectively. The oxygen dependence of the sensors was tested and the following results as showed in figure 5.8.

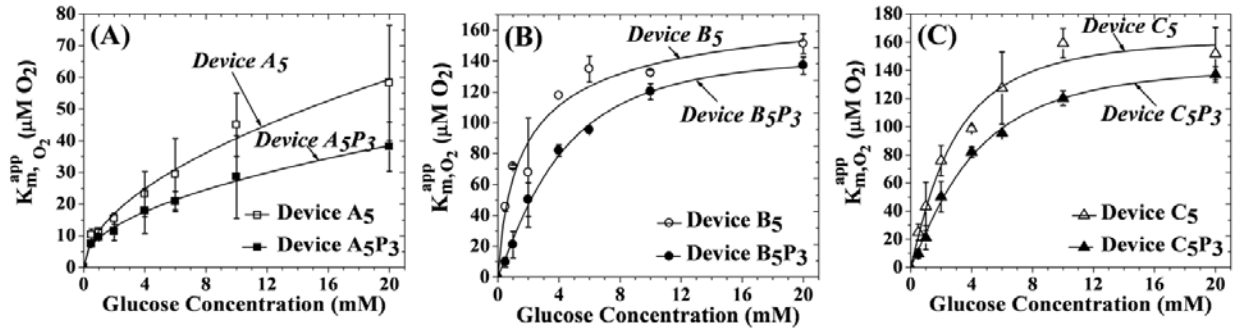


Figure 5.8. This figure showed the K_{m,O_2}^{app} as a function of glucose concentration for sensors containing five bilayers of (A) HA/Fe³⁺, (B) HA/PDDA, and (C) PSS/PDDA semipermeable membranes, before and after deposition of PVA hydrogels, subjected to three freeze–thaw cycles²³.

When we compare figure 5.1, which depicts the saturation of K_{m,O_2}^{app} as a function on glucose concentration, with figure 5.8, it can be determined that with the addition of five bilayers of LBL membranes there is a 65%, 9%, and 5% in the saturation of K_{m,O_2}^{app} for devices A₅, B₅, and C₅ respectively²³. Additionally, with the addition of PVA hydrogels the K_{m,O_2}^{app} is lowered even further by 35%, 12%, and 6% for A₅P₃, B₅P₃, and C₅P₃²³. It was further determined that with the addition of PVA a relative rise in $K_{m,Glu}^{app}$ was observed along with a decrease in K_{m,O_2}^{app} . All of the data obtained was then summarized in table 1 and 2, as shown:

Before PVA				
Sensor	Device Name	$K_{m,Glu}^{app}$ (mM)	LRS (mA/mM*cm ²)	Oxygen slope (mM O ₂ /mM Glu)
Pt/PPD/GOx/HAS/Fe ³⁺	A ₅	11	11	9
Pt/PPD/GOx/HAS/PSS	B ₅	3.3	7	23
Pt/PPD/GOx/PSS/PDDA	C ₅	3	10	23

Table 1: Shows the $K_{m,Glu}^{app}$, LRS, and oxygen slope for sensors made using various semi-permeable outer-membranes. Table 1 summarizes the data obtained during the experiment, indicating Pt/PPD/GOx/HAS/Fe³⁺ to be the best device with a very high $K_{m,Glu}^{app}$, LRS, and low oxygen slope.

After PVA				
Sensor	Device Name	$K_{m,Glucose}^{app}$ (mM)	LRS (mA/mM*cm ²)	Oxygen slope (mM O ₂ /mM Glu)
Pt/PPD/GOx/HAs/Fe ³⁺	A ₅ P ₃	14	4	5
Pt/PPD/GOx/HAs/PSS	B ₅ P ₃	4	4	19
Pt/PPD/GOx/PSS/PDDA	C ₅ P ₃	4	5.6	20

Table 2: Shows the $K_{m,Glucose}^{app}$, LRS, and oxygen slope for sensors made using various semi-permeable outer-membranes. Table 2 summarizes the data obtained during the experiment, indicating Pt/PPD/GOx/HAs/Fe³⁺ to be the best device with a very high $K_{m,Glucose}^{app}$ and low oxygen slope. [LRS= Linear range sensitivity].

5.4. Conclusions:

The optimization of PVA membranes for glucose biosensors required various tests to determine the optimal environment for the PVA. Initially, swelling experiments were carried out to determine the weight change of the PVA as a function of time as well as the saturation weight change as a function of freeze-thaw cycles. This experiment allowed us to determine that as the number of freeze-thaw cycles increased, the percent weight change decreased, with 7 freeze-thaw cycles showing the lowest percent weight change and one freeze-thaw cycle showing the greatest percent weight change. This allowed for the development of an outer membrane which allowed for optimal glucose and oxygen flux to increase $K_{m,Glucose}^{app}$ and decrease K_{m,O_2}^{app} . It was also determined that in order to allow for proper PVA swelling, which allows for optimal glucose flux, PVA needs to be immersed in PBS for three hours.

PVA was then coated onto the glucose biosensors and various batches were fabricated with varying freeze-thaw cycles. It was determined that with the addition of PVA, the response current dropped due to the decreased glucose flux, but the $K_{m,Glucose}^{app}$ and K_{m,O_2}^{app} increased. This indicated that with the addition of PVA, the sensors were able to detect higher concentrations of

glucose as they previously had done and were less dependent on oxygen. It was further concluded that device A₅P₃ was optimal for our sensor design because not only did device A₅P₃ shown the highest sensitivity towards glucose with a high $K_{m, Glu}^{app}$ but also a high LRS, providing the ability to detect wide ranges of glucose, along with a low oxygen dependence.

Chapter 6: Future Outlook

At the end of this project, our glucose sensors had attained a final configuration of Pt/PPD/GOx/HAs/Fe³⁺₅/PVA₃(3 FT cycles). These sensors have the ability to detect the ability to detect increasing concentration of glucose with their high $K_{m, Glu}^{app}$, and low oxygen dependence. Future experiments to further improve linearity, LRS, and ability to detect a wide range of glucose concentrations with low oxygen dependence can be addressed via the incorporation of oxygen storing (myoglobin) and oxygen generating (catalase) species within the outer membrane.

To eliminate the saturation kinetics due to oxygen, oxygen storing (myoglobin) and generating (catalase) enzymes can be used. These will allow us to improve sensor sensitivity and eliminate saturation kinetics. Catalase is an enzyme found in peroxisomes, which catalyzes the oxidation of H₂O₂ to produce water and oxygen¹⁵. The reaction of catalase is as shown: $2H_2O_2 \rightarrow 2H_2O + O_2$. Catalase will immobilized onto the glucose biosensor to generate oxygen to prevent sensor saturation with increasing glucose concentrations. Catalase can be incorporated within the PVA membrane, so not only will it provide the sensor with oxygen but also eliminate any hydrogen peroxide leaking to the outside.

Myoglobin can be used as the oxygen-storing protein. Myoglobin is a globular protein found in high concentrations in muscle and plays an important role in the storage of oxygen. Higher concentrations of myoglobin in muscle allow for greater endurance and allow muscles to worker longer. This is because at low oxygen partial pressure, it will give up its oxygen to prevent anaerobic respiration. These properties can potentially be use to enhance performance of our glucose biosensors. Myoglobin can also be incorporated within the PVA membrane. This

will provide the myoglobin with a ready oxygen supply which it can store, and eventually give up to the enzyme at times when the oxygen partial pressures decline.

Figure 6.1 shows the ultimate configuration of the sensor based on the findings of this project and aforementioned future experiments.

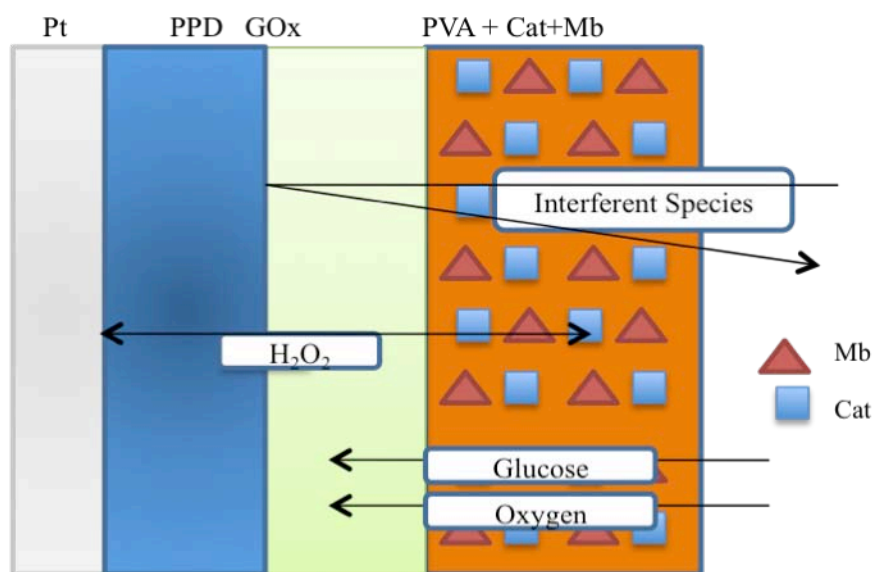


Figure 6.1: Shows the ultimate configuration of the sensor.

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